Characterisation of Lipids from *Litsea graciae* and *Litsea cubeba* Fruits and Their Biological Activities

Jenny Pui Sze Woon (36352)

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Supervisor: Prof. Dr. Fasihuddin Badruddin Ahmad

Resource Chemistry
Department of Chemistry
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak
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Declaration

I hereby declare that no portion of the work referred to in this final year report has been submitted in support of an application for another degree or qualification to this university or any other institution of higher learning.

Jenny Pui Sze Woon

Resource Chemistry

Department of Chemistry

Faculty of Resource Science and Technology

Universiti Malaysia Sarawak

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List of Abbreviations

CHD	Coronary heart disease
DPPH•	2,2- diphenyl-1-picrylhydrazyl radical
EC_{50}	Half maximal effective concentration
GC-MS	Gas Chromatography-Mass Spectrometry
HIV	Human immunodeficiency virus
LC_{50}	Median lethal concentration
PUFAs	Polyunsaturated fatty acids
UV	Ultraviolet

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ABSTRACT

Lipids extracted from the fruits of *Litsea graciae* and *Litsea cubeba* were selected for this study. The samples were extracted using Soxhlet extraction technique and analysed by Gas Chromatography-Mass Spectrometry, and chemical analysis such as acid value and saponification value. The average % (w/w) yield of lipid from *L. graciae* (2.18 % ± 2.06 % for fresh flesh; 3.12 % ± 0.31 % for fresh seed and 20.28 % ± 1.75 % for dried seed powder) and *L. cubeba* (6.17 % ± 1.17 %) was identified. Both lipids have an acid value of 29.05. *L. cubeba* gave saponification value of 108.46 ± 3.24 while *L. graciae* gave 96.31 ± 5.84 and 174.85 ± 3.2 for the Soxhlet extracted flesh and dried seed powder, respectively. Palmitoleic acid was the most abundant unsaturated fatty acids present in the fresh flesh of *L. graciae* (26.55 %) and fresh seed of *L. cubeba* (20.35 %). For the saturated fatty acids composition of *L. graciae*, palmitic acid was mostly found in fresh flesh (15.20 %), lauric acid in both fresh seed (75.59 %) and dried seed powder (80.26 %). Stearic acid was the most composition in *L. cubeba* fresh seed (50.31 %). Biological activity such as brine shrimp lethality assay and antioxidant assay was carried out. Both *L. graciae* (LC_{50} = 639.04 ppm and 777.75 ppm for solvent soaking extraction and Soxhlet extraction respectively) and *L. cubeba* (LC_{50} = 779.71 ppm) oil showed to have a low toxicity as their LC_{50} (50 % mortality concentration) is over 500 ppm. EC_{50} (50 % effective concentration) values for *L. graciae* lipids (solvent soaking extracts = 3.09 µg/mL and Soxhlet extracts = 2.01 µg/mL), and Soxhlet extracted *L. cubeba* lipid (4.91 µg/mL) calculated were lower than that of ascorbic acid standard (11.12 ± 3.47 µg/mL).

Keywords: *Litsea* spp.; lipid yield; brine shrimp lethality assay; antioxidant assay

ABSTRAK

Lipid yang diekstrak daripada buah Litsea graciae dan Litsea cubeba telah dipilih untuk kajian ini. Sampel tersebut telah diekstrak menggunakan teknik pengekstrakan Soxhlet dan dianalisis dengan Kromatografi Gas Spektroskopi Jisim, dan analisis kimia sepeti nilai asid dan nilai penyabunan. Hasil peratusan lipid (w/w) daripada L. graciae (2.18 % ± 2.06 % untuk isi segar; 3.12 % ± 0.31% untuk biji segar dan 20.28 % ± 1.75 % untuk serbuk biji kering) dan L. cubeba (6.17 % ± 1.17 %) telah dikenal pasti. Asid palmitoleik asid merupakan asid lemak tidak tepu yang paling banyak terdapat dalam isi segar L. graciae (26.55 %) dan biji segar L. cubeba (20.35 %). Bagi komposisi asid lemak tepu dalam L. graciae, asid palmitik paling banyak terdapat dalam isi segar (15.20 %), asid laurik terdapat dalam kedua-dua biji segar (75.59 %) dan serbuk biji kering (80.26 %). Asid stearik merupakan komposisi yang terbanyak (50.31 %) dalam biji segar L. cubeba. Kedua-dua lipid mempunyai nilai asid 29.05. L. cubeba memberikan nilai penyabunan 108.46 ± 3.24 manakala L. graciae memberikan 96.31 ± 5.84 dan 174.85 ± 3.2 untuk isi dan serbuk biji kering yang diekstrak secara Soxhlet masing-masingnya. Aktiviti biologi seperti ketoksikan anak udang marin dan esei antioksidan telah. Kedua-dua lipid L. graciae (LC_{50} = 639.04 *ppm dan 777.75 ppm untuk pengekstrakan pelarut rendaman dan pengekstrakan Soxhlet masing-masing) serta L. cubeba* *(LC_{50} = 779.71 ppm) menunjukkan ketoksikan yang rendah disebabkan LC_{50} (50 % kepekatan kematian) mereka melebihi 500 ppm. Nilai EC_{50} (50 % kepekatan berkesan) untuk lipid L. graciae (ekstrak rendaman pelarut = 3.09 µg/mL dan ekstrak Soxhlet = 2.01 µg/mL), dan lipid L. cubeba diekstrak daripada Soxhlet (4.91 µg/mL) dikira kurang daripada piawai asid askorbik (11.12 ± 3.47) .*

Kata Kunci: Litsea spp.; peratusan lipid; ketoksikan anak udang marin; esei antioksidan

Chapter 1

Introduction

1.1 Background of Study

Over 2500 species and 32 genera in the Lauraceae family are widely distributed in the subtropics and tropics regions of South and North America and Asia (Simić et al., 2004). *Litsea* spp. is one of the important genus in the Lauraceae family. The genus composed by 622 species of trees and shrubs are mainly distributed in tropical and subtropical low land forests in Asia, South and North America, Australia and New Zealand (Sri-Ngernyuang et al., 2007; Agrawal et al., 2011). *Litsea* plants have a smooth and reddish brown bark; small flower, alternate leaf with naked buds. Currently, 54 species of *Litsea* species, for example, *L. paludosa, L. cubeba, L. resinosa*, *L. graciae*, *L. glutinosa* has been reported in Malaysia (Corner, 1988) with a high distribution in the East Malaysia region such as Kapit and Sematan in Sarawak and Ulu Kinabatangan, Nabawan and Pensiangan area in Sabah. Roots, leaves and barks of *L. glutinosa* and *L. cubeba* have been extensively studied to determine the chemical composition of the essential oil (Si et al., 2012) and are widely used in traditional medicine in healing muscle pain and even coronary heart disease (Hwang et al., 2005; Wang and Liu, 2010).

Lipids are hydrophobic organic compounds that are insoluble in water; indeed lipids are readily soluble in the organic solvents (Murphy, 2005), such as ether and alcohol. They are commonly named according to their physical properties as fatty or waxy compounds. Lipids include fatty acids, wax and phospholipids (Smith et al., 2013). Among all the lipids available, the market demand for polyunsaturated fatty acids (PUFAs) is on the rise currently; however the supply of PUFAs is in a scarce and meanwhile, at this moment, genetically engineered oilseed plants are found to be an alternative resource of PUFAs

(Alonso, 2000). Hence, these fatty acids have become one of the interests of study in order to classify them based on their degree of unsaturation.

Various researches on the leaves, barks and seeds extracted from *Litsea* plants regarding their biology activities have been investigated. These include the cytotoxicity activity in *L. akoensis* (Tanaka et al., 2009), fungicidal activity and antibacterial activity of *L. cubeba* (Yang et al., 2010; Zhang et al., 2012). Antioxidants have high contents of polyunsaturated fatty acids and thus play an important role in human diet (Pokorný, 2007). Recently, the demand for natural antioxidants is on its rise as the commercialized synthetic antioxidants selling in the market have been reported containing toxicity-causing substances and are carcinogenic (Cirilo and Lemma, 2012). Among the *Litsea* spp., *L. monopetala* bark extracts (Arfan et al., 2008) and leaves of *L. coreana* var. *lanuginose* or Hawk tea (Jia et al., 2013) already been determined to show strong antioxidant properties. Hence, there should be more manufacturing of natural sources antioxidants products. However, the information of the antioxidant bioassay of the *Litsea* spp. fruits against DPPH• (2,2-diphenyl-1-picrylhydrazyl radical) is very limited.

Majority of the studies done by other researchers have been focusing on the bark and leaves of the *Litsea graciae* and *Litsea cubeba* plants and their chemical composition found in them. Although the fruits of these two species are widely consumed and/or used in the daily life, there is little information on the saponification and acid values, and antioxidant activities of lipids found in the fruits. Therefore, this present experiment aims at the characterization of lipids extracted from selected *L. graciae* and *L. cubeba* fruits based on the chain length (carbon number) and the degree of unsaturation (number of double bond presence). Furthermore, the brine shrimp lethality assay indicating the cytotoxicity level of the lipids is also another field to study.

The objectives of the study are:

a. to extract and characterise lipids found in the fruits of selected *Litsea* spp.
b. to determine the antioxidant and cytotoxicity activities of the extracted lipids in *Litsea* spp. fruits.

Chapter 2

Literature Review

2.1 *Litsea* spp.

Plants from Lauraceae family are found to have a significant contribution in improving human health and also act as an agent for cancer chemoprevention in traditional medicine (Lin et al., 2007). The *Litsea* genus contains evergreen of deciduous trees or shrubs belonging to the Lauraceae family. The swampy and watery areas in Malaysia are the habitat for about 54 species of *Litsea* plants (Wyatt-Smith, 1999). Various researchers have been used most of the parts of the *Litsea* plants in their studies. Research done had shown that parts of the *Litsea* plants containing a very high source of biological-active compounds. For instance, the leaves of *Litsea japonica* consist of flavonoids (Lee et al., 2005); the fruits of *Litsea cubeba, Litsea graciae* and *Litsea monopetala* are rich in essential oils (Amer and Mehlhorn, 2006); sesquiterpenes and fatty acids in most *Litsea* spp. (Agrawal et al., 2011).

The plant of *Litsea akoensis* is a medium-sized, evergreen tree. The fruit appears in round shaped with cup fruit tray. The stem bark of this species was used by Chen et al. (1998) in identifying the *in vitro* cytotoxicity. Its bark stem showed a significant cytotoxic activity against HT- 29 cancer line cells. Other than that, the bark also contains a high level of syringaldehylde (Tsai et al., 2000). The extracts from this species show that it has anti-inflammation properties and with a dose of 25 μg/mL extraction, the manufacture of nitric oxide in the LPS-induced microphage test can be inhibited (Lin et al., 2007).

Litsea cubeba sp. is an evergreen tree found in Southeast Asia, Japan, Taiwan and China (Huang et al., 2008) with commonly known names as 'May Chang' in China and Taiwan, and 'Medang Padang' in Sarawak. The fruits produced are small in size and in black colour. The bark of *L. cubeba* has been widely utilised as an oriental medicine in treating atopic eczema (Zhang et al., 2012). Works of extracting and analyzing the essential oil of *L. cubeba* also been carried out by Luo et al. (2005) and Yang et al. (2010). The essential oil was employed in producing perfumes, vitamin A manufacturing and flavor enhancer in foods due to its unique smell of citrus-pepper mixtures (Zhang et al., 2012). The seed of it was used to produce bio-oils which are more suitable for subsequent refining compare to the microalgae's bio-oil production (Wang et al., 2010).

'Engkala' or 'Pengolaban' is the common name known for *Litsea graciae* by the natives in Sabah and Sarawak. It has big-sized fruits compared to fruits of other *Litsea* spp. The fruits are pink in colour when it ripe. The Muruts in Sabah used inner barks of *L. garciae* as a traditional medicine in treating joint dislocation and sprain (Kulip, 2003).

The *Litsea glutinosa* species has a high dispersity in Philippines, India and Borneo. It is commonly known as 'Brown Bollywood' or 'Indian laurel'. The appearance of the fruits is round in shape with the colour range between purple-brown to shiny black. The fruits of *glutinosa* were extracted to obtain essential oil and later employed in rheumatism (Amer and Mehlhorn, 2006). The natives practiced the bark and leaves as an emollient and demulcent and also mild astringent for dysentery (Das et al., 2013). The sequential extract of the leaf contributed to medicine industry by relieving cough and respiratory disorders (Pradeepa et al., 2011).

Litsea verticillata plant has oval berries with shallow cup fruit tray. It was mainly employed in the determination of anti-HIV activity by studying the lignans separated (Hoang et al., 2002). Research has been done on the leaves and twigs of this plant in order to identify the type of biologically active compounds in it. It is showed that the plant was rich in sesquiterpenes (Zhang et al., 2003), which inhibited the HIV-1-replication.

The sources of knowing *Litsea resinosa* is limited as there is not much research being done on this species. However, studies done showed there was a big amount of essential oil constituents present in it (Ahmad et al., 2005). The methanol extracts of stem and root of *L. resinosa* were reviewed and gave significant scavenging activity with EC_{50} of 35.48 mg/L. The hexane extracts from the stem and chloroform extracts from the inner barks showed potent antibacterial assay on gram negative bacteria *Escherichia coli* and gram positive bacteria *Bacillus subtilis* respectively with their inhibition on it (Wong, 2014).

L. monopetala or *L. polyantha*, is a small and medium-sized plant with a height of 12 m has stalked and oblong shaped leaves. The common names of it are Medeasak (Arfan et al., 2008), Pojo and Kakuri. According to Ghosh and Sinha (2010), the tree bark has a unique odour. Arthritis and diarrhea can be treated with barks. This plant also has been reported as a traditional analgesic. Study prior to *L. polyantha* has determined that this species is a priceless medicinal plant in folklore medication (Ghosh and Sinha, 2010).

Other popular genera belonging to this family are *Cinnamomum and Persea*. The *Cinnamomum* spp. are frequently known as cinnamon, for example *Cinnamomum zeylanicum*. According to Simić et al. (2004), the essential oil containing *trans*-cinnamaldehye and eugenol extracted from *C. zeylanicum* posses a strong antifungal property. *C. camphora* produced camphor that can be employed to heal colds and chills (Lee et al., 2006), diarrhea, nervousness and also synthesised into mothballs (Lin et al., 2007). Meanwhile, avocado (*Persea americana*) fruit tree is the common species found in *Persea* spp. The leaves of the *P. americana* were utilised in pharmacological evaluation with its acetone extracted. The concentration of the acetone extracted more than 150 μg/mL showed a powerful vasolidating activity. This plant was widely used in regulating menstruation cycle (Odo, 2013).

Table 2.1: Litsea spp. and their usage

Type	Part Used	Usage	Reference
L. akoensis	Stem bark	Anti-inflammatory activity	Lin et al. (2007)
L. cubeba	Bark	Atopic aczema treatment	Zhang et al. (2012)
	Seeds	Microalgae's bio-oil production	Wang et al. (2013)
	Fruits	Essential oil manufacturing	Yang et al. (2010)
	Root, leaves	Healing muscle pain	Hwang et al. (2005)
L. graciae	Inner barks	Joint dislocation and sprain treatment	Kulip (2003)
L. glutinosa	Fruits	Essential oil extraction	Amer and Mehlhorn (2006)
	Barks	Mild astringent foe dysentery	Das et al. (2013)
	Leaves	Relieving cough	Pradeepa et al. (2011)
L. verticillata	Leaves, twigs	HIV-1 replication inhibition	Zhang et al. (2003)
L. coreana	Leaves	Antioxidant assay	Jia et al. (2013)
L. monopetala	Leaves, root	Skin disease treatment	Ghosh and Sinha (2010)
	Barks	Arthritis and diarrhea treatment	Ghosh and Sinha (2010)
L. resinosa	Inner barks	Antibacterial activity	Wong (2014)
	Stem, root	Antioxidant assay	Wong (2014)

2.2 Lipids

Lipids or fats and oils are basically grouped into two categories, polar and non-polar lipids, in terms of their chemical compositions. Polar lipids such as phospholipids and glycolipids, while non-polar lipids like the mono-, di- and tri- glyceraldehydes, waxes and isoprenoid-type hydrocarbons (Belotti et al., 2013). Lipids are commonly represented by fatty acids. The fatty acid content in seeds of several *Litsea* sp. such as *L. glutinosa*, *L. laeta*, *L. angustifolia* and *L. lanuginosa* distributed in North-East India were studied by Kotoky et al. (2007); the result measured ranged between 19.1-58.6 % (w/w). The fatty oil extracted from these species is analysed with gas chromatography and it is found that the dominant fatty acid is the lauric acid which brings benefit to human health and contributes in personal care industry. Monolaurin converted from the lauric acid gives advantages towards the human body because of its antibacterial, antiprotozoal and antiviral properties (Enig, 1998). Despite the major fatty acid, oleic, stearic, palmitic, myristic and linoleic acids are found in all the species (Kotoky et al., 2007).

2.2.1 Fatty Acids Compositions

The physical properties of both triglycerides and fatty acids refer to the number of double bonds (unsaturated) and chain length (Mittelbach and Remschmidt, 2004). The type of fatty acids composition and percentage depends on the species of plant and their growth manners (Atabani et al., 2012). According to Akbar et al. (2009), there are three main categories of fatty acids found in a triglyceride, which are saturated (Cn: 0), monounsaturated (Cn: 1) or unsaturated and polyunsaturated fatty acids (Cn $\geq$ 2). Saturated fatty acids are made up of single-chain hydrocarbons (Table 2.2), for example lauric acid, palmitic acid and stearic acid. Monounsaturated fatty acids (Table 2.2) such as myristoleic acid, palmitoleic acid and oleic acid have one double bond present in the hydrocarbon chain. Polyunsaturated fatty acids (PUFAs) or polyenoic fatty acids contain two or more double bonds (Table 2.2) like stearidonic acid, dihomo-γ-linolenic acid and α-eleostearic acid.

Table 2.2: Types of fatty acids

Type	Common name	Systematic name	Stuctural Formula	Shorthand Description
Saturated Fatty Acids	Lauric acid	n-Dodecanoic acid	$CH_3(CH_2)_{10}COOH$	12:0
	Palmitic acid	n-Hexadecanoic acid	$CH_3(CH_2)_{14}COOH$	16:0
	Stearic acid	n-Octadecanoic acid	$CH_3(CH_2)_{16}COOH$	18:0
Mono-unsaturated Fatty Acids	Myristoleic acid	Tetradec-9-enoic acid	$CH_3(CH_2)_3CH=CH(CH_2)_7COOH$	14:1
	Palmitoleic acid	Hexadec-9-enoic acid	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$	16:1
	Oleic acid	Octadec-9-enoic acid	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$	18:1
Poly-unsaturated Fatty Acids	Stearidonic acid	Octadecatetraenoic acid	$CH_3CH_2(CH=CHCH_2)_4(CH_2)_3COOH$	18:4
	Dihomo-γ-linolenic acid	Eicosatrienoic acid	$CH3(CH_2)_4(CH=CHCH_2)_3(CH_2)_5COOH$	20:3
	α-Eleostearic acid	Octadeca-9,11,13-trienoic acid	$CH3(CH_2)_8(CH=CH)_3(CH_2)_4COOH$	18:3

Oils with high amount of saturated fatty acids are known to bring negative impact towards human lipid concentration (Beardshell et al., 2002). Therefore, the intakes of oil containing more PUFA and less saturated fat as unsaturated fatty acids are found to be heart healthy.

2.2.2 Polyunsaturated fatty acids (PUFAs)

Fatty acids with two or more double bond are known as polyunsaturated fatty acids. Generally, they are named in abbreviated form as X:YnZ, where X stands for the number of carbon atoms of the chain, Y is the number of double bonds, and Z represents the position of the first double bond (unsaturated) counted from the methyl end (the n system of numbering) (Alonso, 2000). Vegetable oil with high degree of unsaturation tends to have high freezing point (Akbar, 2009) due to their thermal stability. According to Dutta et al. (2014), oils containing three double bonds and above tend to show lower thermal and oxidative stability. Among the examples of PUFAs, omega-3 and omega-6 fatty acids are widely known due to their applications for daily consumption in human diet.

2.2.2.1 Omega-3 (ω-3)

The omega-3 fatty acids have their first double bond positioned at the third carbon counted from the methyl end group. Studies on omega-3 showed that sufficient intake of omega-3 can help in improving human health. Serum triglycerides, inflammation and arrhythmia can be reduced with the consumption of omega-3 fats (Geelen, 2004). Furthermore, omega-3 PUFAs lower the heart rate and blood pressure and thus prevent the infection of cardiovascular disease. However, the possibility of getting coronary heart disease (CHD) can only be decreased with the intake of plant-based n-3 PUFAs when there is a little accessibility of seafood-based n-3 PUFAs (Mozaffarian et al., 2005). Long chain omega-3 fatty acids (Table 2.3) for instance docohexaenoic acid (DHA), docosapentaenoic acid (DPA) and eicosapentaenoic acid (EPA) are generally found in fish oils; Short chain n-3 PUFA (Table 2.3) such as α-linolenic acid (ALA) that can be converted to DHA and EPA is found in some vegetable and plant oils.

Table 2.3: Omega-3 fatty acids

Chain length	Common name	Systematic name	Formula	Shorthand Description
Long chain	Docohexaenoic acid	Docosa-4,7,10,13,16,19-hexaenoic acid	$CH_3CH_2(CH{=}CHCH_2)_6(CH_2)COOH$	22:6
	Docosapentanoic acid	Docosa-7,10,13,16,19-pentaenoic acid	$CH_3CH_2(CH{=}CHCH_2)_5(CH_2)_4COOH$	22:5
	Eicosapentaenoic acid	Eicosa-5,8,11,14,17-pentaenoic acid	$CH_3CH_2(CH{=}CHCH_2)_5(CH_2)_2COOH$	20:5
Short chain	α-linolenic acid	Octadeca-6,9,12-trienoic acid	$CH_3(CH_2)_4(CH{=}CHCH_2)_3(CH_2)_3COOH$	18:3

The recommended volume of intake of EPA per day from the plant oil is about a spoonful or around 10 g and this amount is considered sufficient for human diet (Abbadi et al., 2004). ω-3 fatty acids proven to have significant impact on the brain in terms of structure and role of the brain. Investigation of ALA deficiency suggested this phenomenon altered the configuration and degrade the function of brain membrane (Bourne, 2004).

2.2.2.2 Omega-6 (ω-6)

Linoleic acids (LA) and arachidonic acids (AA or ARA) are the examples of omega-6 fatty acids (Table 2.4). The ω-6 has their first double bond at the sixth carbon counted from the methyl end group. Studies have determined the n-6 plant-based PUFAs or ω-6 extracted from vegetable oils has the potential in reducing the infection or CHD due to their significant impact towards insulin sensitivity or serum lipids especially the linoleic acid (Mozaffarian et al., 2005).

Table 2.4: Omega-6 fatty acids

Common name	Systematic name	Formula	Shorthand Description
Linoleic acid	9,12-Octadecadienoic acid	$CH_3(CH_2)_4(CH{=}CHCH_2)_2(CH_2)_6COOH$	18:2
Arachidonic acid	5,8,11,14-Eicosatetraenoic acid	$CH_3(CH_2)_4(CH{=}CHCH_2)_4(CH_2)_2COOH$	20:4

2.2.3 Extraction of Fatty Acids

According to Atabani et al. (2012), there are three techniques that have been utilised in extracting fatty acids or the oil: enzymatic extraction, solvent extraction and mechanical extraction. Among the methods, mechanical and solvent extractions are the commonly used.

Enzymes are utilised in the enzymatic oil extraction to extract fatty acids from ground seeds. This eco-friendly technique is gaining attention as no volatile organic compounds will be produced. The addition of alkaline protease in this aqueous extraction enhanced the performance of the method. Nevertheless, this process required a long time to obtain the yield (Atabani et al., 2012).

The extraction from *Idesia polycarpa* using mechanical expeller in mechanical extraction indicated a high yield of production (Yang et al., 2009). However, the design of mechanical extractor only matched for specific seeds only and thus affects the yield (Atabani et al., 2012).

Solvent extraction means the addition of liquid solvent will eliminate one constituent from a solid. There are three ways of methods using this type: hot water extraction, Soxhlet extraction, ultrasonication method. The liquid solvent chosen must be selective and have low viscosity such as n-hexane. Despite the selection of solvent used, temperature, particle size and agitation of the solvent are the other factors manipulating the rate of solvent extraction. Small particle size gives more sample-solvent interaction; solubility of the test sample reduced when the temperature decreased; and agitation of solvent boost the transfer of substance from the particles' surface. (Atabani et al., 2012).

2.2.4 Determination of chemical compositions

Chemical parameters such as the saponification value and acid value were used in measuring the chemical compositions found in lipids (Inekwe et al., 2012).

The amount of mg potassium hydroxide needed to saponify 1 g of oil gives the meaning of saponification value (Al-Bachir, 2014). It also measures the mean of molecular weight of fatty acids present (Inekwe et al., 2012). High saponification number indicates more alkali